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A CRITICAL REVIEW OF THE BACTERIOLOGY
OF HUMAN AND RAT LEPROSY

S. B. WOLBACH, M.D.

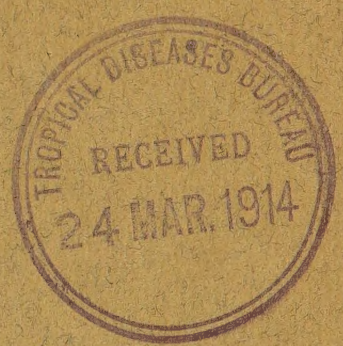
AND

JAMES A. HONEIJ, M.D.

(From the Department of Bacteriology, the Harvard Medical School)

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A CRITICAL REVIEW OF THE BACTERIOLOGY OF HUMAN AND RAT LEPROSY.*

S. B. WOLBACH, M.D., AND JAMES A. HONEIJ, M.D.

(From the Department of Bacteriology, the Harvard Medical School.)

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A thorough review of the literature dealing with efforts at the cultivation of the bacillus of leprosy was undertaken preliminary to attempts to confirm some of the more recent and promising results. The results of this study of the literature were so interesting and suggestive, that we thought it best to publish our attempt to arrange and analyze the whole. We have endeavored to include all data descriptive of the bacteria isolated, and of the methods employed, so that further recourse to original publications will not be necessary by those interested in this subject. Many descriptions are so meager that it has been impossible in some instances to accurately group the organisms isolated.

In all, twenty-six different authors have described cultures obtained from leprosy which demand consideration, and these

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cultures may be divided into four groups, the largest of which is the group of partially acid-fast or acid-resistant diphtheroid organisms — the Babes-Kedrowski type. This group includes fourteen authors.

The next largest group includes five authors, and is that of acid-fast organisms, which produce yellow or orange colored colonies. Organisms of this type were first isolated by Rost, and first carefully studied by Clegg.

The third group includes three authors, and is that of anaërobic acid-fast organisms, first reported by Ducrey, but probably more often identified with the name of Campana.

The fourth group is that of acid-fast bacilli which do not produce colored colonies. Five authors, of which Karlinski was the first, have claimed to have obtained organisms of this type. Duval's recent work is the most important dealing with this type of organism.

A number of isolations have been discarded because of the obviously saprophytic nature of the organism obtained; for instance, the liquefying non-acid-fast organism of Van Houtem, and the conidia-producing streptothrix of Deycke-Pascha. We have endeavored to include only those authors whose methods, as described, bear evidence of competency, and the chief claim for consideration in a review of this nature lies in the facts that many of the authors are truly competent bacteriologists, and in the similarity of results obtained by many of them.

We have included separately, abstracts of all papers reporting the cultivation of the bacillus of rat leprosy, because of the similarity of the varieties of bacilli obtained to those found in human leprosy.

BACILLI OF THE DIPHTHEROID TYPE.

Bordoni-Uffreduzzi.¹ — Cultures were obtained from the bone marrow removed twenty-four hours post-mortem from a case of leprosy. The culture medium was peptone glycerin blood serum; the exact method of preparation is not stated; optimum temperature 36° to 37° C.; transfers grew readily upon glycerin agar; growth appeared in seven days.

The color of the colonies on the peptone glycerin blood serum medium is light yellow; the serum is not liquefied. Growth takes place in the water of condensation, which remains clear. The first generation would not grow at room temperature, but finally after six months, small colonies developed on glycerin gelatine. Growth on ordinary agar agar was very slow; potato and bouillon were absolutely unsuited.

The colonies are described as grayish white, circular, thick in the middle, and thin at the periphery with serrated edges. The colonies tend to remain isolated for a considerable time — eight to ten days; finally they coalesce.

The morphology is given as thin, straight or bent, and curved rods of different lengths, one to five or six μ long. The majority of the bacilli have terminal swellings, occupying one-third of the total length of the bacillus. The smallest bacilli are coccoid in appearance, while the largest reach double the average length and are apparently two bacilli joined. These long forms have club swellings at both ends. In addition to the club endings, the bacilli contain clear spaces. The organism is non-motile.

The bacilli resist decolorization with alcohol after staining with aniline water fuchsin or gentian violet. This method of staining demonstrates a capsular substance about some of the organisms. This material, which is said not to be a true capsule, decolorizes when treated by the Koch-Ehrlich staining method. After decolorization with alcohol or treatment with the Koch-Ehrlich staining method, nearly all the bacilli show one to four clear spaces. The author compared the staining reaction of his cultures with cultures of tubercle bacilli, and could find no absolute difference in the results.

A large number of guinea-pigs, rabbits, and mice were inoculated subcutaneously, intravenously, intraperitoneally and into the anterior eye-chamber, with entirely negative results. A number of controversial articles, regarding this publication were written by Babes and Baumgarten. In Bordoni-Uffreduzzi's² final reply he contributes nothing to the above description of the bacillus isolated by him.

Babes.³— A culture was obtained from a case of leprosy, eight hours post-mortem, upon glycerin blood serum. The colonies grew fairly rapidly and profusely, and were whitish in color. The bacilli were very thin; $.3\ \mu$ in diameter; curved with swollen ends; often surrounded by a "contour line" as if encapsulated. There were also much larger bacilli, with deeply staining clubbed ends and granules. The bacilli decolorize by Ehrlich's method, though the large clubbed ends resist decolorization by acids somewhat. (The briefness of this description is accounted for by the fact that this bacillus was one of many studied by Babes in a research of metachromatic granules.)

A more complete description of Babes' cultures appeared in *Les Bactéries*.⁴ At this time cultures upon glycerin beef serum, glycerin agar and sometimes on ordinary agar had been obtained from three cases of leprosy and from various organs. At body temperature, colonies appeared eight days after inoculation; no growth occurred on gelatine and potato. The colonies are somewhat similar to those of the diphtheria bacillus, but are larger. On glycerin serum, they show yellowish white, elevated plaques with irregular or serrated edges surrounded by a very delicate, transparent zone. They produce a slightly aromatic odor. They grow upon the surface as well as in the depths of the medium.

The morphology is given as somewhat similar to the diphtheria bacillus; slender, often curved rods with tapering and clubbed ends, $.2$ to $.3\ \mu$ thick. They stain less well with ordinary stains than the diphtheria bacillus and contain metachromatic granules. They are surrounded by a zone, as if encapsulated. In old colonies, very large forms are found, $.5$ to $1\ \mu$ thick. The bacilli decolorize by Ehrlich's method, although the clubbed ends and granules remain slightly stained. They were not pathogenic for mice, rabbits, guinea-pigs, monkeys, fowls, and goats.

Babes⁵ claims priority over Bordoni-Uffreduzzi for the cultivation of diphtheroids from leprosy. He has succeeded in obtaining cultures from twelve cases, but no further description of the cultural and morphological characteristics is

given. He⁶ distinguishes now between two types of colonies, which differ in morphology from each other. The first was of the type already described. The second were smaller and more transparent colonies, composed of smaller and more acid-resistant bacilli, and resemble more the leprosy bacillus in tissues. Babes expresses the opinion that this second form goes over into the first.

Babes⁷ also discusses the relationship to leprosy of the similar cultures isolated by others, with particular reference to the inoculation experiments of Kedrowski; see below.

Gianturco.⁸ — A culture was obtained from one case, from a non-ulcerated cutaneous nodule. Growth was visible upon glycerin agar after seven days' incubation at 37° C. Subcultures grew readily, and the colonies are visible after two days. Upon glycerinated blood serum the growth is luxuriant and the colonies become confluent. Growth takes place slowly upon ordinary agar, though the colonies remain small in comparison with those upon glycerin agar. No growth occurs on other ordinary media. The colonies on agar media are grayish white in color; upon blood serum they have a yellowish tint. The form is circular with serrated edges, and new colonies take origin at the periphery of older ones, thus producing a grouping, likened to the appearance of trifolium leaves. The growth does not penetrate the media. No growth occurs at room temperature (18° to 24° C.), but the bacilli survive this temperature for long periods.

The morphology varies with the medium. Upon glycerinated blood serum and upon ordinary agar, the bacilli are shorter and thinner than those from glycerin agar, and resemble leprosy bacilli from tissues. Beaded bacilli and bacilli with club-shaped and spherical ends are frequent, as well as bacilli with oval spore-like bodies which stain deeply. In old cultures, most of the bacilli present deeply staining spots, separated by faintly staining spaces, and have terminal swellings at one end; rarely at both. The terminal swellings sometimes reach a large size, and finally are ellipsoidal in

shape, staining very deeply and often are detached. Gianturco regards these bodies as spores.

The bacilli are Gram positive, stain well with carbol fuchsin, aniline oil gentian violet, and methyl violet, and resist decolorization by the Koch-Ehrlich and Baumgarten's method. They also resist decolorization with glacial acetic acid, oxalic acid, and ten per cent nitric acid in alcohol. On the other hand, they stain with safranine, vesuvine, and eosine. The author notes an oscillating motion of the bacilli in hanging drop preparations, but from his description it is evident that the movements were Brownian in character.

Levy, E.⁹ — A culture was obtained from a living case of a mixed type of leprosy. A non-ulcerated nodule was thoroughly excised and the tissue squeezed out upon glycerin agar streaked with sterile human blood, and incubated at 37° C. Colonies appeared after fifteen days, in two out of ten tubes inoculated. Sub-cultures grew readily on glycerin agar and blood serum; a slight growth occurred on gelatine at 20° to 22° C. The colonies are grayish white with irregular borders, from the serrated periphery of which new colonies take origin. Old cultures are wrinkled and present an appearance like three to four weeks' old glycerin agar cultures of mammalian tubercle bacilli. The appearance of cultures on blood serum and on Loeffler's blood serum is similar to that on glycerin agar. Growth in bouillon is poor and gives rise to numbers of small floating particles, which eventually sink to the bottom, leaving the bouillon clear. The morphology of the bacillus is described as thin, slightly bent rods, about four μ long and 1.3 thick, and non-motile. They have a tendency, in the colonies, to form chains or rope-like masses twenty to thirty μ long. The bacilli often have a swollen or clubbed end. These swollen ends are 2.3 to 3.4 μ thick; the opposite end is often tapering. In addition to the clubbed swellings, the bacilli contain metachromatic granules. The bacilli are Gram positive, and after staining with carbol fuchsin, they

withstand decolorization for a few seconds with absolute alcohol and with dilute sulphuric acid. They decolorize by the Fränkel-Gabbett method of staining for tubercle bacilli, although the clubbed swellings and granules retain the red stain. Occasionally branching forms are found, and these are demonstrable in stained and unstained preparations. The arms of the branches are of equal or unequal length, and the division may occur at the swollen end of a bacillus; at the junction of the two branches there is often a meta-chromatic body. Numerous mice, guinea-pigs, and rabbits were inoculated subcutaneously, intraperitoneally, intravenously and into the anterior eye chamber. None of these animals showed any evidence of infection. Several of each were killed at the end of one, two, and several weeks; no lesions were found.

Spronck.¹⁰ — Cultures were obtained from two cases: 1, From an autopsy two hours post-mortem, from a skin tubercle and from the bone marrow, of many tissues tried. 2, From an excised cutaneous lesion from a tuberculous case of leprosy. An attempt from a third case, using an excised cutaneous nodule, failed. The medium employed was a neutral glycerin potato medium. The exact method of preparation is not given. Transfers grew readily on Loeffler's blood serum and agar agar at 37° C. There was no growth at room temperature nor on transfers to potato, although the original isolations were obtained on potato. This the author explains by assuming that the tissue contained the necessary nutrient substances. The colonies on serum-containing media are yellowish white. The growth is slow, and they become visible to the eye in about ten days. They are circular in outline, with irregular edges, which send out prolongations. In ordinary bouillon, and in human and horse blood serum, and in exudates and transudates from man, very slight growths were obtained. Profuse growth appeared in simple fish bouillon. Cultures grow best in oxygen, although growth does take place under anaërobic conditions. The size is given as slightly longer and thicker

than the leprosy bacillus in tissue, and the bacilli decolorize more rapidly with twenty-five per cent nitric acid, after staining with carbol fuchsin, although the exact staining reaction is not given. The bacilli are not motile, and do not produce spores. No pathogenicity was observed for guinea-pigs, white mice, goats, and pigeons.

Spronck believes his culture to be identical with those of Bordoni-Uffreduzzi, Gianturco, Babes, Levy, and Czaplewski. While the morphology of this culture is not described, Spronck states, in the conclusions, that it is a diphtheroid. He made many agglutination tests with serum from leprous and non-leprous patients, and found that it was very unusual for a non-leprous serum to agglutinate his bacillus at dilutions of 1-30 and 1-40; while the serum from leprous patients agglutinated at dilutions of 1-60 to 1-1000.

Czaplewski.¹¹ — One culture was obtained upon a medium composed of sheep's blood serum with six per cent of glycerin, inspissated and sterilized (Bordoni-Uffreduzzi's medium). Material was obtained from the nasal secretion and from the discharge of an ulcerated nodule in the throat. This was spread over the medium with a platinum spatula, the tubes sealed with paraffin and incubated at 37° C. Colonies were seen after the first week. The first tube was examined at the end of twelve days, at which time he found among colonies of other bacteria, grayish yellow colonies, from which he made transfers to other flasks of glycerin serum.

The colonies of his culture are grayish yellow in color; roughly circular with somewhat serrated edges. The under surface of the colonies is slightly raised; the consistency crumbly. They grow well on all ordinary media, with the exception of potato; growth is especially profuse on Loeffler's blood serum. The growth in general resembles that of the diphtheria bacillus. With very favorable media and rapid growth, the consistency of the colonies becomes softer and the color lighter. Contact cover-glass preparations show an arrangement of the bacilli in bundles and chains, somewhat similar to the arrangement in anthrax colonies. The best

growth takes place at 37° C., and in the presence of oxygen, although a fairly good growth is obtained on gelatine at room temperature. The description of the colonies upon agar is almost identical with that of the Bordoni-Uffreduzzi culture; brownish gray colonies; thick in the center with irregular serrated edges. Bouillon cultures were very slightly clouded; pellicle formation was not observed. The growth settled to the bottom as a fairly coherent, compact sediment. The bacilli stain well with most stains, particularly with methylene blue, fuchsin and gentian violet, although they stain less intensely with Loeffler's methylene blue than does the diphtheria bacillus, and the same is true with water diluted alcoholic methylene blue. They retain the stain by Gram's method. With tubercle bacillus stains, there is a certain degree of resistance against decolorization with alcohol, and with mineral acids, although not so marked as with the tubercle bacillus. The following methods of decolorization leave the bacilli stained in varying degrees of intensity; the least energetic come first:

- 5% sulphuric acid.
- 30% nitric acid.
- 5% sulphuric acid followed by alcohol.
- 30% nitric acid followed by alcohol.

In young cultures only do the bacilli themselves show resistance against decolorization. In old cultures it is only the granules and clubbed swellings that retain the stain.

The morphology is described as of a straight or slightly bent bacillus, the length of which varies with the rate of multiplication. In general, the size and thickness depends upon the character of the medium; dimensions are not given. The bacilli are much larger on blood serum media than on glycerin agar. They are particularly large in the slowly growing gelatine cultures. The ends of the bacilli are pointed or blunt, knobbed or club shaped. Regularly pointed ends are found only in young cultures. Frequently the ends are found blunt or terminating in clear vacuoles, which are considered to be results of plasmolysis. In old

cultures, the bacilli are often knobbed or club shaped. The old cultures also show numerous granules, similar to those found in tubercle bacilli and in diphtheria bacilli. In very old cultures, the bacilli disintegrate, leaving behind only deeply staining granules in masses. In bouillon, peculiar forms occur with large, cylindrical swellings in the middle or ends. Similar forms are found in gelatine cultures and here occasional branching forms occur.

Inoculation experiments with rabbits (subcutaneous, intraperitoneal, and intravenous), guinea-pigs (intraperitoneal and lumbar), and mice (subcutaneous and intraperitoneal) were negative. Czaplewski regards his cultures as identical with those isolated by Bordoni-Uffreduzzi and Gianturco.

Teich.¹² — The first culture was obtained from an aseptically excised nodule from a patient with tubercular leprosy. Four cultures were obtained from four other cases, using nasal secretions from three and blood from an incised nodule from the fourth; these five cultures were all identical. The medium employed for the first culture was glycerin agar; the surfaces of some tubes were smeared with blood obtained at the operation. For the subsequent isolations a potato medium flowed with five per cent soda solution (carbonate?) and an alkaline potato agar were used. This last medium was prepared by grinding up potatoes and expressing the fluid, which was allowed to stand over night, decanted, boiled, and filtered. In the filtrate, there was dissolved one per cent of peptone and two per cent agar. The whole was neutralized with soda solution, until red litmus paper was colored a distinct blue. This medium was tubed and sterilized by fractional sterilization. None of the cultures, including the one made from the excised nodule, were pure from the start, and pure cultures were only obtained by subsequent plating. The colonies of the diphtheroids finally obtained are meagerly described as whitish colonies with serrated edges, reaching a thickness of three to four millimeters in a few days; later they become confluent. On the potato agar, growth was extremely rapid and in

twenty-four hours covered the surface with a three to four millimeters thick, light brown membrane; the water of condensation remained clear or slightly clouded. The staining reaction is given as follows: Loeffler's methylene blue stains uniformly and intensely. Carbol fuchsin followed by ten to fifteen seconds' treatment with three per cent solution of hydrochloric acid in alcohol leaves the bacilli well stained. Longer treatment with acid decolorizes the bacilli and leaves behind stained granules. The morphology of the bacilli is very imperfectly given. They are described as rods showing a great variety of forms, varying in length and thickness, and with swellings sometimes at the ends and sometimes in the middle; in general, resembling the forms exhibited by the diphtheria bacillus; no motility was observed; dimensions are not given.

Teich concludes that his cultures are in all probability identical with those of Bordoni-Uffreduzzi, Babes, Czaplewski, and Spronck. He lays particular stress on the pleomorphism of the bacillus, and the facts that on favorable media it assumes delicate forms like those of the leprosy bacillus in tissue, while on less favorable media and in old cultures it assumes diphtheroid characters with involution forms.

Barannikow.¹³ — This is a preliminary communication. The composition of the media used, details of methods and descriptions of cultures are not given. The following statements are made: non-motile and partially acid-fast rods were cultivated from early nodules in two cases of leprosy. The nature of the medium is more important than the temperature of incubation. Human brain and transudates properly treated give good media. Growth takes place in thirty-six to forty-eight hours on agar or gelatine. Growth at room temperature occurred after four days. The bacilli are said to correspond with the description of the cultures of Babes, Bordoni-Uffreduzzi, Gianturco, Levy, Czaplewski, and Spronck. The bacilli in general resemble the tubercle

bacillus and the diphtheria bacillus. Dimensions are not given.

Two ^{14, 15} later papers are similar to the first in the absence of exact data and observations, and the conclusions of the author may be summarized as follows: The micro-organism of leprosy undergoes a complicated cycle of development. The different forms possess different pathogenicities for animals. In one stage, the bacillus loses entirely its acid-fast properties. At times, washing with water will decolorize preparations stained with methylene blue. Cultures were obtained from a nodule which had been dried for ten days, although the dry material gives rise to colonies much later than attempts from fresh tissue or blood. In old cultures, the organism takes on actinomyces-like forms. In the absence of exact data it is impossible to decide upon the value of Barannikow's observations. It seems probable, however, that his cultures do resemble those of the authors he names.

Kedrowski.¹⁶ — Cultures were obtained from three cases. The medium was placenta extract agar made as follows: Fresh human placenta was ground up in a meat grinder and extracted from eighteen to twenty-four hours with one and a half to three times its volume of distilled water, and this extract was sterilized by passing through a Chamberland filter. The amount of water employed was determined by the nature of the placenta, its succulence and blood content. This sterile extract was added to nutrient agar agar in order to obtain a solid medium. The proportions are not given. The first isolation was obtained from an excised skin nodule. He inoculated tubes which were sealed and incubated at 37° C. Colonies appeared in from two to five days. The second case was from a twelve-year-old boy with follicular and perifollicular leprosy. The material used for culture was necrotic material obtained by aseptically opening the follicles. Three different attempts gave the same culture. From the third case, excised tissue was employed. Colonies appeared on the third day. Two attempts at cultivation from this

case gave the same result, which showed some differences from that of the first two. The cultures from the first two grew profusely. The first generation would only grow upon placenta extract agar, but afterwards the organisms grew well upon glycerin and plain agar agar, gelatine, and bouillon.

The surface colonies on agar agar showed the central elevation and serrated borders observed by Bordono-Uffreduzzi and Czaplewski. Bouillon cultures were slightly clouded and yielded a thin pellicle. The growth finally settled to the bottom, leaving the bouillon almost clear. In gelatine, growth was slow, and took place in the upper part of the stab and upon the surface; there was no liquefaction. On inspissated egg yolk the bacilli grew rapidly and became yellowish white in color; the water of condensation was not clouded. Staining reactions: The bacilli stain with lal aniline dyes, but show considerable resistance to decolorization after staining with carbol fuchsin. They would completely withstand energetic treatment with five per cent sulphuric acid. If the preparations were stained for twenty-four hours by Ehrlich's or Ziehl's method, the bacilli would withstand brief treatment with five per cent sulphuric acid. The most marked resistance to decolorization was observed in young cultures ten to fourteen hours old. Old growths decolorize completely with the exception of granules which retain the stain.

The morphology of the bacilli varies considerably with the culture medium employed. Upon inspissated egg yolk and blood serum the bacilli were short and thick; almost coccoid in appearance. On agar agar and placenta extract agar the rods were long and thinner, resembling bacilli in leprous lesions. In cultures the bacilli show parallel arrangement. The ends of the bacilli are often pointed, and in old cultures, fourth to fifth day, long bent forms are found. In still older cultures, involution forms are common, and swollen and clubbed ends and vacuoles are commonly present. The long forms contain many metachromatic granules, which retain the fuchsin stain after treatment with acid. Dimensions are not given. Kedrowski says the bacteria are unquestionably

motile, particularly in short individuals in agar agar and blood serum cultures, but in later publications no mention of motility is made. The cultures from case three gave smaller and colorless colonies, somewhat similar to those of the influenza bacillus, but in the course of time, after five or six generations, the colonies approached in appearance those of the other cases. They would not grow in gelatine, and in bouillon did not form the surface pellicle. In young cultures the bacilli are short, but soon become longer; they show marked granulation and clubbed endings. The granules and clubbed endings show marked resistance to decolorization by acid, after the carbol fuchsin staining. This culture is on the whole more resistant to decolorization than the others. Young growths resist energetic decolorization with five per cent and ten per cent sulphuric acid; old ones decolorize with the exception of the metachromatic granules. This culture showed a great tendency to long or thread forms; occasional branching is seen. Kedrowski considered all his cultures to be of the same organism.

Another paper¹⁷ contains the results of animal experiments conducted during a period of nine years. In March, 1900, a rabbit was inoculated into the substance of the brain with the culture from case three, described above. From time to time (dates not given) intraperitoneal inoculations with the same culture were made. The rabbit died in November, 1901. This rabbit showed extensive lesions of a granulomatous nature with giant cells and epithelioid cells, filled with enormous numbers of acid-fast leprosy bacillus-like organisms. From this rabbit he obtained in culture acid-fast organisms and three types of diphtheroid organisms. Subsequent inoculation experiments of rabbits and white mice convinced Kedrowski that these cultures were all stages of the same organism, and that the acid-fast property was acquired in the course of residence in the animal body. The loss of the acid-fast property was observed in old cultures, while the non-acid-fast or partially acid-fast bacilli became acid-fast in tissues. In view of Kedrowski's conclusions of

the non-permanent characteristics of the semi-acid-fast organisms, upon which he based his division into three types, it does not seem advisable to include his descriptions because in general the cultures are very similar to those described in the first paper and, as he says, after cultivation they all acquire similar characteristics.

The acid-fast organism, which was cultivated, grew very slowly upon placenta extract agar. The colonies appeared in three and a half to four weeks. They were minute, almost opaque, gray, which grew out of the tissue placed upon the medium. They grew slowly, and in two to two and a half weeks reached a diameter of 1.5 to two millimeters. Their consistency was soft and they readily emulsified in water.

The morphology is given as of thin, straight or slightly curved rods. They stain poorly with ordinary aniline dyes. After staining by the Ehrlich or Ziehl-Nielsen methods they resisted ordinary decolorization with ten to twenty-five per cent sulphuric acid and thirty per cent nitric acid, followed by eighty to ninety per cent alcohol for fifteen to thirty seconds. Transfers upon freshly prepared media (placenta agar) gave a moist growth, similar to the so-called homogeneous tubercle bacillus cultures of Arloing-Courmont.

This paper is discussed by Babes.¹⁸ He criticises the origin of the original culture, because there was but one colony, and the possibility of contamination could not be excluded. The possibility of the rabbit, from which Kedrowski obtained his four varieties of bacilli, having become infected with a variety of tuberculosis is suggested. He also suggests the possibility of Kedrowski having carried along this tubercle bacillus in his cultures of non-acid-fast organisms. Babes believes the lesions described by Kedrowski are more like those produced after inoculation with certain forms of tuberculosis (avian). Babes insists on the following, which he presents in the form of conclusions:

1. That the lepra bacillus is often less acid resistant than the tubercle bacillus; it loses its resistance more easily, namely, after treatment with antiformin. After such treatment it stains with simple aniline stains much easier than does the tubercle bacillus.

2. That the diphtheroids cultivated from leprosy first described by Babes were obtained in pure cultures and from the internal organs, and that similar bacilli have been found in the leprosy-like disease of rats (Dean).
3. That these bacilli are agglutinated by leprosy serum and in two instances an extract of these bacilli with leprosy serum has yielded a complement binding system.
4. That some of the attempts at inoculation with leprosy upon monkeys, rats, mice and dancing mice, and in the anterior eye chamber of rabbits, have afforded good evidence of success.
5. That the bizarre and questionable results obtained by Kedrowski and other investigators demand energetic investigation of the question of the cultivation and pathogenicity of the leprosy bacillus, and that these problems are by no means solved.

Klitin.¹⁹ — Cultures were obtained from excised cutaneous nodules from four cases of the tubercular type of leprosy. The media employed are not described but are designated by letters and evidently represent well-known material. Besides the regular media, which Klitin designates by letters, he used gelatine, agar, and bouillon, with the additions of human blood and human ascitic fluid. Grape sugar from .3 to .5 per cent and glycerin four to six per cent were also used. The agar media contained four per cent of agar; the gelatine media twenty per cent of gelatine. The most favorable media were "R.A." and "S.A." Aërobic methods only were used. The optimum temperature was 37° to 38° C. Tubes inoculated with the tissue emulsified in normal saline solution gave higher percentages of growth than those inoculated with solid pieces of tissue. The first growth on "R.A." and "S.A." became visible in five days. Transplants grew readily upon these media and also, but less luxuriantly, upon "M.P.A." with glycerin, and in glycerin gelatine. The latter was not liquefied. Other media which were suitable for sub-cultures were Loeffler's blood serum, cooked egg albumin, potato and several fluids designated as "R.M.P.B.," "M.P.B." with grape sugar, and "S.M.P.B."

The colonies on "R.A." and "S.A." are whitish, translucent, round, convex and have elevated centers which are more opaque than the periphery. They are easily removable

from the surface of the medium and diffuse readily in water. Transplants on most media develop in from twenty-four to forty-eight hours. After two to three days, colonies become confluent and less transparent. On Loeffler's blood serum they assume a yellowish tint; on potato they form a brownish gray layer. On the surface of coagulated egg albumin the colonies are soft, gray in color, and at the end of a week take a yellowish tint. In liquid media, "M.P.B." with glycerin a copious sediment is formed which, on shaking, clouds the medium. The medium in general remains clear. The consistency of this culture in liquid media is ropy; no surface pellicle is formed; carbohydrates are not fermented. On glycerin agar growth is poor and the colonies remain discrete, grayish white, and translucent.

The morphology varies upon different media. Dimensions are not given. On "R.A." and "S.A." the bacilli are thin and in length vary from three-tenths to seven-tenths the diameter of a red blood corpuscle. In general the morphology is given as similar to that of *B. tuberculosis*, but the bacillus is shorter and more granular. It is straight or slightly bent; the extremities may be of equal thickness, but sometimes one end is much thicker than the body of the rod; occasionally both ends show terminal swellings. Longer bacilli may have one end pointed and the other end swollen. They usually contain two to three thickenings in the middle of the rod and occasionally rods appear as if composed of several short elements, which resist decolorization by acids, while the connecting substance takes the counterstain. The bacilli on Loeffler's blood serum are thicker than the above. The irregular staining was not so prominent and clubbed endings less common. Growth upon white of egg was similar to that on Loeffler's blood serum, while in gelatine cultures the rods are very thick and of the same length as upon "R.A." and "S.A." The bacilli from all cultures are arranged in parallel package-like masses.

Staining reaction: After staining by the methods of Ehrlich and Ziehl the bacilli resist decolorization for two to three seconds with five per cent nitric acid and two per cent

sulphuric acid. They resist decolorization by alcohol for a long time. They are Gram positive. Loeffler's methylene blue and ordinary methylene blue in two to three seconds stain the bacilli faintly. An aqueous solution of gentian violet gives a clear, strong stain. After prolonged cultivation the resistance to decolorization by acids is nearly completely lost, and they become decolorized with one per cent acid solutions and show much less resistance to decolorization with alcohol. After counterstaining with methylene blue the rods stain pale blue but contain one to two minute red stained granules. Klitin did not study his cultures longer than four months, although he showed that the cultures remained visible from nine to ten months. The acid-fast granules were still present in these old cultures. The bacilli were said by Klitin to be motile, although his description leaves much doubt on this point, and it is quite possible that Brownian motion was all that was observed. No serum tests were made. Cultures were inoculated into rabbits of all ages, from three to six weeks up to fully grown rabbits, and into guinea-pigs. Twenty-four-hour cultures from glycerin agar were used, and the methods of injection were intraperitoneal, intravenous, and subcutaneous. The young rabbits died at the end of about one week with acute infection of the peritoneum and pleura, and other signs of an acute infectious process. The older rabbits lived about four months and showed necroses in liver and granulomatous lesions in various organs, which microscopically were composed of connective tissue and resembled the leprous nodules in the skin of man! Klitin recovered the organisms in cultures from these animals and regarded them as identical with those obtained from man.

Levy, F.²⁰ — Cultures were obtained from one case, that of a young man with nodular lesions on the face, forehead, and arms. The material used was a nodule excised from the arm. The media employed were six per cent glycerin veal broth as used for the cultivation of the tubercle bacillus (Nocard and Roux); a simple beef broth and a broth made

from pancreas (method of preparation not stated). The first cultures were obtained in the glycerin veal broth, incubated at 37° C. The first evidence of growth appeared in forty-eight hours as a granular deposit on the sides and bottom of the test-tubes. After a few days a white surface pellicle formed. The broth itself remained clear. Transplants to simple meat broth and milk gave growth without change in the appearance of the medium other than the formation of a pellicle and a sediment. Transfers to melted gelatine at 38° C. gave an abundant growth. The appearance of the cultures was similar to that in broth, except that wavy strands of growth extended through the medium and remained attached to the sides of the test-tube. Stab cultures in gelatine kept at 24° to 25° C. gave growth, visible in seven days; no liquefaction occurred. Surface colonies on gelatine and on agar agar were whitish, circular in outline with serrated edges and a raised, opaque center. Old colonies became dirty yellowish or grayish. On Heyden's agar, growth is abundant, and the colonies send out finger-like projections, which Ducrey likens to Marguerites. On inspissated blood serum there was an increased transparency of the medium about the line of growth, but no liquefaction. Other media, which gave growth, not characteristic, were veal blood serum (simple and solidified) and human ascitic fluid. Growth on potato was slow and appeared as a thin, soft scum with a yellowish tint. Growth on all media was best with free access of oxygen. Under anaërobic conditions growth occurred but less luxuriantly.

Cultures were also obtained directly from this patient upon slabs of red beet prepared and sterilized after the fashion employed in making potato media. One out of four tubes inoculated gave growth. In a subsequent experiment two out of four tubes inoculated with tissue from three different nodules from this patient gave growth. Sub-cultures on red beet, incubated at 38° C., gave minute grayish colonies in thirty-six to forty-eight hours. Later the colonies became confluent and formed a viscous whitish layer. Transference of these cultures to veal broth in Erlenmeyer flasks gave

much better growth than the broth in test-tubes. This is explained by the larger surface presented to the air and freer access of oxygen.

The morphology in the first generations in glycerin veal broth is given as that of a straight or slightly curved bacillus two to six μ long; often with clubbed extremities. In old cultures in other media the clubbed ends are much more striking. The bacillus when stained is granular in appearance and has much the same morphology as the diphtheria bacillus. The arrangement, however, is different, and is that of radiating masses, similar to that described by Bordoni-Uffreduzzi.

Staining reaction: Exact data are not given. The first generations on red beet and glycerin broth were said to be acid-fast and alcohol-fast. During cultivation the bacilli lose their acid-fast properties and gradually acquire the staining reaction of the diphtheria bacillus. No animal experiments nor serum tests were made.

Bayon.²¹ — The cultures studied came from an excised nodule. The media used were placenta extract agar and horse serum-neutrose, to which was added two per cent of ground up smegma bacilli according to Twort's method. The cultures are described as acid-resisting diphtheroids. After staining for half an hour with warm carbol fuchsin it cannot be bleached by one per cent sulphuric acid in five seconds. On injection into the peritoneum of a mouse it acquires acid-fast properties in the abdominal glands, without markedly changing its morphology. In subsequent pure cultures it resisted bleaching with twenty-five per cent sulphuric acid. A later experiment from the same patient yielded a "filamentous, branching, absolutely non-acid-resisting organism, which grew quite rapidly within three days on the culture medium used. It was a milky white, moist, smooth growth." This organism was injected into rats and became acid-fast in the nodule that developed at the site of the inoculation. Sub-cultures developed an acid-fast rod, identical in morphology with Hansen's bacillus.

"The resulting culture was practically identical in all details with the moist, wrinkled, milky white acid-fast culture isolated by Kedrowski in Moscow, only that it took much longer to grow in sub-cultures; in some cases as much as six months. A similar culture isolated from a mouse and rabbit which had been injected with the acid-fast strains of human leprosy bacillus was non-acid fast, filamentous, and branching. After fourteen months' growth on Dorset's egg serum at 37° C. it changed into an acid-fast rod. The change occurred gradually, and took first the form of acid-fast granules in the bacilli. These granules gradually increased in size and quantity, so that eventually many acid-fast bacilli were found mixed up with the original culture." These papers contain no exact data.

In a general review of leprosy Bayon²² states that his culture is identical with that of Kedrowski, and that it acquired acid-fast properties after injection into animals. He states that Kedrowski's view "that the parent form of Hansen's bacillus is a filamentous, interlacing, branching, non-acid-fast microörganism is correct and that this pleomorphic property is shared by several other acid-fast bacteria." No new cultural data are given.

Williams.²³ — From lepers in Persia and India Williams claims to have cultivated an organism which he calls *Streptothrix leproides*. This organism exhibits the following different stages:

- "a. A non-acid-fast streptothrix in the mycelial stage and producing acid-fast rods."
- "b. A non-acid-fast diphtheroid bacillus producing also acid-fast rods."
- "c. An acid-fast bacillus which is but the broken down stage of a streptothrix."
- "d. An acid-fast mycelium."

All inoculations were made from non-ulcerated lesions. The medium used for the isolations was a fluid composed of "Lemco" broth (composition not given), without the addition of salt or peptone, two hundred and fifty cubic centimeters, distilled water, two hundred and fifty cubic centimeters, and milk, fifty cubic centimeters. This medium differed from

the original medium used by Major Rost in the substitution of distilled water for an equal quantity of distillate obtained by blowing steam over rotten fish.

The non-acid-fast streptothrix in the mycelial stage produced visible growth in ten days. In the first culture nests of acid-fast bacilli were present, lying in the meshes of the mycelium. These diminished in numbers on sub-culture and after three sub-cultures were not found. When sub-cultures were made on potato broth an orange yellow surface membrane was formed.

The non-acid-fast diphtheroid bacillus appeared as a flocculent precipitate lying at the bottom of the tubes after seven days' incubation. "This was seen to consist of masses of lepra bacilli lying in the zoogloea substance. Extended observation showed that the basis of the membrane was a streptothrix varying as to its acid-fastness." On tubes of "Lemco" glucose agar, without salt or peptone, sub-cultures showed colonies in eight to ten days. Single colonies were very small, dense in the center, with wavy irregular margins. Both acid-fast and non-acid-fast bacilli were found in these colonies, but the former gradually died out after a number of sub-cultures. When these non-acid-fast diphtheroid organisms were mixed with amebæ they became acid-fast in forty-eight hours after ingestion by the amebæ.

"The acid-fast bacillus phase, which is a broken down stage of a streptothrix:" This phase appeared after sub-culture from B in the form of a yellow, coherent growth in the bottom of Rost's medium. "This growth was strongly acid-fast, but by careful study the stages from the non-acid-fast diphtheroid to the acid-fast stage could be made out." Finally this phase was made to grow on solid media, which produced yellow, sticky colonies or a yellow wrinkled membrane. It is pleomorphic.

"The acid-fast mycelial stage:" This was obtained once directly from a leper and also as a development from the non-acid-fast mycelial stage. The growth from the leper was obtained on Dorset's egg medium. It appeared as a streptothrix which was acid-fast, then non-acid-fast, and

finally broke up into acid-fast and non-acid-fast bacilli. "In sub-culture on ordinary agar it became a purely non-acid-fast bacillus. By putting it back into Rost's original medium it then became a beautiful acid-fast filamentous streptothrix."

Williams says: "Regarding pigment formation in these growths I should like to say that pigment formation varies so much, even in the same streptothrix, when the conditions are slightly altered, as to make the growth of practically no value in differentiating between these organisms." A detailed description and more accurate data are not given in this paper. Williams' culture in its acid-fast bacillary form after subcutaneous injection into guinea-pigs produced lesions "somewhat resembling leprosy, with large numbers of cocco-bacilli in the connective tissue cells."

Duval.²⁴ — From one case of leprosy, out of many studied, in connection with the cultivation of acid-fast bacilli, a non-acid-fast streptothrix was obtained, which is said by the author to resemble in "every respect" Kedrowski's and Bayon's description of the non-acid-fast stage of their cultures. Duval's culture has never shown the least degree of acid-fastness. It grows slowly upon favorable media at 37° C. and produces a moist film-like growth "similar to *B. influenza*." On ordinary nutrient agar and bouillon no growth occurs. The sugars are not fermented. "In stained preparations from Loeffler's blood serum it appears as a diphtheroid bacillus, which is indistinguishable from *B. xerosis*, while from glycerin agar it is filamentous and often branched. The culture is non-pathogenic." Duval refers to this organism in previous papers, but this is the most complete description.

ACID-FAST CHROMOGENIC CULTURES.

Rost, E. R.²⁵ — The character of the cases and tissue used in making the cultures are not given. The medium employed was a salt-free broth, first obtained by distilling beef extract. Later, he prepared his medium by passing superheated steam through bottles containing pumice stone

soaked in beef extract. The steam is condensed and collected in sterilized Pasteur flasks, and when cooled is ready to inoculate. One ounce of beef extract will give two thousand cubic centimeters of nutrient fluid. A solid medium was prepared by the use of agar (proportions not given) previously rendered salt-free by dialysis. According to Rost the bacillus of leprosy grows on the surface of solid media as a white or very slightly yellow beady growth. The colonies appear in two days. Later, the colonies become yellow or brick red. In the fluid medium "the characteristic appearance is a curly, white, stringy, heavy deposit at the bottom of the tubes, which is hard to shake up, but when shaken appears as a curly, white, stringy shred in the tubes." "Cultures were obtained from a great many different cases."

The bacilli were acid-fast, retaining the stain of carbol fuchsin after treatment with twenty-five per cent nitric acid. "It is more irregular than the tubercle bacillus, and not curved, and is somewhat smaller. It contains small oval spores within itself, which are wholly refractile, and the end of the bacillus may be open where some have presumably escaped. It has a beady appearance due to the presence of these oval spores. Like the tubercle bacillus it may grow out into cultures, into long, branching filaments, but there are often oval spores, separate in the cultures, and these may be alone visible at times." No animal experiments are given.

Williams.²³ — The organism which Williams claims to have cultivated has already been described under the diphtheroid group. In view of the fact that he describes an acid-fast bacillary strain, producing yellow colonies, notice is again made of his work in this place.

Clegg, Moses T.;²⁶ Currie, Brinkerhoff, and Holman;²⁷ Currie, Clegg, and Holman.²⁸ — The technic employed was devised by Clegg. In the last reference the authors summarize the independent work done by each. The present

status of the research begun by Clegg is that acid-fast bacilli have been grown in symbiosis with cholera bacilli and amebæ at least sixteen times from fifteen cases of leprosy. Pure cultures of acid-fast bacilli have been obtained seven times. Method: The medium employed for the cultivation of amebæ consists of agar, 20; sodium chloride, 3; extract of beef, 3; and water, 1000. The preparation is the same as for ordinary nutrient agar. The final reaction should be one per cent alkaline to phenolphthalein. The extract of beef may be omitted. Plate cultures of this medium are inoculated with material containing amebæ and bacteria, such as feces or the scum which forms upon the surface of water, after the addition of alkaline bouillon in the proportion of 1 to 100 and twenty-four to forty-eight hours' incubation at room temperature. When growth of amebæ and mixed cultures are so obtained, it is possible to obtain the amebæ growing in symbiosis with a single culture. This is accomplished first by inoculating plates of the above medium with the desired bacterium, so that several concentric rings of growth form. The mixed culture of amebæ and bacteria is placed in the center. As the amebæ spread over the plate they pass through the bacterial rings, and repetition of this procedure will finally result in the exclusion of all bacteria but the one desired. The leprosy material, which included material obtained at autopsy (spleen) and excised nodules from living cases is added to a mixed culture of amebæ and cholera bacilli. After incubation for one week, multiplication of acid-fast bacilli may be demonstrated. The cultures are transferred about once a week. Pure cultures are obtained by heating the mixed cultures at a temperature of 60° C. for half an hour, then transferring to plain nutrient agar. The acid-fast bacilli survive this treatment, while the amebæ and cholera bacilli do not. Many tubes must be inoculated from each case, for only a small percentage show multiplication of the acid-fast bacilli, and many of the cultures that do start die before they reach the third generation. In the cultivation of amebæ it is essential to have the right temperature, which

is that temperature which will maintain the proper rate of growth between bacteria and amebæ. An optimum temperature for the bacteria will enable them to grow too rapidly, and thus overgrow the amebæ. The following description of the cultures is taken from the last named reference which, bearing the name of Clegg, should be authoritative:

On ordinary bouillon: "In young cultures, a fine flocculent growth adhering to the sides of the tube, from which attachment it sends out stalactites. No clouding of the fluid; no pellicle formation. On shaking the tube a thick, tenacious sediment arises from the bottom. In very young cultures the appearance in bouillon is quite similar to a *B. pestis* or a streptococcus culture. Old cultures differ from the above description in having a heavy wrinkled surface growth, which in time becomes of a golden yellow color. Glycerin bouillon: The same as ordinary bouillon, except that the organism grows more rapidly and more luxuriantly in this medium than in the last named. Glycerin agar: Growth on this medium varies in color from a canary yellow to a deep orange, the latter shades being more commonly met with, but slight shades of color variations, independent of any apparent cause, are commonly noted. The growth extends slightly beyond the track of the needle stroke, and varies from a slimy to a granular appearance. Surface may be smooth and shining, or rough and nodular, but never truly wrinkled, as is noted in some of this group. Edges of the culture are raised, smooth, sharply defined. Growth is always moist, but this varies somewhat with the reaction of the media. On media just neutral, cultures are apt to be drier and more granular, while on fully alkaline media the growth is more moist, even to the point of being slimy. Plain agar: Growth much thinner on this medium than on that containing glycerin and other nutrient media. Spreads little beyond the track of the needle stroke (this depends, however, somewhat on the moisture of the medium). Except that it is less luxuriant the culture is of the same appearance as that noted on glycerin agar. Ordinary

nutrient agar: Growth heavier than on plain agar, but not as heavy as on glycerin agar, of light yellow to reddish yellow (orange) color, confined to the track of the needle stroke. Surface usually smooth, but sometimes nodular from the heaping up of colonies. Dorsett's egg medium: Growth invariably of a deep orange shade of yellow, none of the 'strains' showing the lighter shades of yellow on this media. Growth is confined to the path of the needle stroke, or spreads very little beyond it; very dry, 'mealy,' granular in appearance, showing neither wrinkling nor glistening of surface. Borders clearly defined; slightly raised above the level of the media. No liquefaction of media at the end of a month's growth. Potato (neutral in reaction): Growth confined to the path of the needle stroke, of a deep orange-yellow color, dry, 'mealy' in character, surface rough from the coalescence of numerous small colonies, but without any tendency to show wrinkling. Dunham's peptone: Fluid clear and devoid of growth of any character, except at the bottom, where a yellowish sediment occurs, which sends up flocculent threads, which float in media. At the time these notes were taken, no pellicle had formed on the media. Glucose broth (in fermentation tubes): A heavy, slightly wrinkled yellow pellicle covers the surface of the fluid, and adheres to the sides of the glass in the aërobic portion of the tube. No growth in the anaërobic portion of the tube. No gas is produced. Lactose, mannite, and saccharose broth in fermentation tubes: Identical in appearance to glucose-broth culture. Blood serum (Loeffler's): Organism spreads considerably beyond track of the needle stroke, probably due to the moisture of the medium; color of a light orange yellow shading into canary yellow at the edges of the culture. Surface moist, smooth, glistening; growth slimy and tenacious. In our experience at this station blood serum is not liquefied by this organism, even at the end of a month's growth. Nutrient gelatine (stab culture): A heaped-up growth occurs on surface surrounding point of inoculation; very slight growth along the path of the needle stab; lightness of growth in depths of media as compared to surface,

apparently being due to the air-loving propensities of the bacillus, as growth becomes progressively lighter as it proceeds down the needle stroke. No liquefaction of the media occurs."

Morphology: The bacillus is non-motile. The size depends upon the nature of the medium upon which the organism is cultivated. The length varies from one to five μ .

Stained with the method of Nielsen the shortest forms stain uniformly as do many of the long forms. In many instances certain portions of the bacilli stain more deeply than others. In young cultures the center of the rods stain more deeply than the poles. Other individuals present a beaded appearance, due to alternating deep and lightly stained areas. The deeply stained areas may be situated in the poles. The majority of young bacteria are straight or curved rods; long, slim forms grew, but branching and spore-formation did not occur. Staining reaction: The bacilli stain faintly with the ordinary alkaline stains. Young cultures when stained with hot carbol fuchsin do not lose the stain when treated with Gabbett's solution. They also retain it after being washed continuously for three minutes in absolute alcohol. When treated for two minutes with a five per cent solution of hydrochloric acid in alcohol, a large number lose the fuchsin stain. There are others, however, which are not affected. Old cultures decolorize more quickly than young, though they resist Gabbett's solution and absolute alcohol.

Animal experiments: Pure cultures produced lesions in guinea-pigs, but not in white rats and monkeys. The lesion in guinea-pigs, after subcutaneous inoculation, consisted of a diffuse thickening and induration with loss of hair.

Microscopical examination shows the formation of "richly cellular granulation tissue, consisting almost exclusively of irregular spindle-shaped young connective tissue cells." The cells contain numerous nuclei and giant cells were found. Bacilli were numerous in this tissue.

Serum reactions: The serum of a horse immunized during three months with repeated injections of live cultures of

four strains produced marked clumping with all of the cultures isolated in dilutions of 1 to 1000.

Duval.²⁹ — Duval succeeded in growing leprosy bacilli by the method of Clegg. Material for his experiments consisted of excised nodules, organs obtained at autopsy, and pus from a subcutaneous abscess occurring in a case of leprous fever. The bacilli were also obtained upon a special medium which was prepared as follows: "the rind was carefully removed from the fruit portion of fully matured bananas, every precaution to avoid contamination being used, and large blocks of the fruit, after slanting one surface with a sharp knife, were introduced into suitable sterile glass cylinders, provided at the bottom with cotton plugs, saturated in sterile distilled water. Sterile one per cent solutions of tryptophane, cystein (made from protein), and leucein were next prepared and a portion of each poured upon and allowed to saturate the banana." The sterility of these solutions was obtained by passage through Berkefeld filters. Another medium was prepared by adding five cubic centimeters of the above mentioned substances to melted tubes of two per cent nutrient agar, adjusted to 1.5 per cent alkalinity to phenolphthalein. Both the banana and the agar, to which cystein and tryptophane were added, proved excellent media. The optimum temperature of growth is 32° C. Light seemed to accelerate the growth. Growth was obtained from four cases. Colonies grew from the first inoculations, and are described as semi-translucent, grayish white. In a later paper³⁰ the color of the colonies is given as at first grayish white, but after several days as distinct orange yellow. In this second paper other media are described, composed of albumin and trypsin. "Egg albumin or human blood serum is poured into sterile Petri dishes, and inspissated for three hours at 70° C. The excised leprous nodule is then cut into thin slices, two to four millimeters in thickness, which are distributed over the surface of the coagulated albumin. By means of a pipette the medium thus seeded with bits of tissue is bathed in a one per cent sterile solution of trypsin,

care being taken not to submerge the pieces of leprous tissue. Sufficient fluid is added to moisten thoroughly the surface of the medium. During incubation more trypsin solution is added from time to time as evaporation necessitates. The first few generations are transferred to a similar medium, but after three or four generations the bacilli may be grown without difficulty upon glycerin serum agar, prepared as follows: twenty grams of agar, three grams of sodium chloride, thirty cubic centimeters of glycerin, and five hundred cubic centimeters of distilled water are thoroughly mixed, clarified, and sterilized in the usual way. To tubes containing ten cubic centimeters of this material is added in proper proportion a solution of unheated turtle muscle infusion. Five hundred grams of turtle muscle are cut into fine pieces and placed in a flask with five hundred cubic centimeters of distilled water. This is kept in the ice-chest for forty-eight hours and then filtered through gauze to remove the tissue. The filtrate is then passed through a Berkefeld filter for purposes of sterilization. By means of a sterile pipette five cubic centimeters of the muscle filtrate are added to the agar mixture, which has been melted and cooled at 42° C. The tubes now are thoroughly agitated and allowed to solidify in the slanted position." Finally the cultures were made to grow upon ordinary blood agar media and eventually upon ordinary agar.

Cultural characteristics: The arrangement of the bacilli in the colonies is like that of the tubercle bacillus, in masses of parallel bacilli. The morphology is like that of leprosy bacilli in tissues. The bacilli are straight or curved, and stain irregularly. They vary greatly in length and thickness; some are short and thick, almost coccoid; others are long and slender, and contain metachromatic granules and alternating light and dark stained areas. Long filamentous bacilli are encountered with blunt and swollen ends; shorter forms with pointed ends are also found. Dimensions are not given.

Staining reaction: The bacilli are fully as acid-fast as those found in human tissues, but less so than the tubercle bacillus. They resist decolorization with Gabbett's solution and the usual treatment with weak acids and alcohol: thirty

per cent nitric acid, followed by ninety-five per cent alcohol decolorized them after a time; not sufficient to decolorize the tubercle bacillus.

Animal experiments: Lesions were produced in Japanese waltzing mice. The pathological histology is given as identical with that of human leprosy.

Two inoculation experiments with monkeys, *Macacus rhesus*, are recorded with autopsies. Both monkeys showed disseminated infection. The gross and microscopical findings are reported as similar to those in human leprosy.

Duval and Wellman.³¹ — The authors describe as new a medium made from human placenta extract and agar. This medium is essentially the same as that described by Kedrowski. Duval and Wellman give the following method of preparation: "the fresh human placenta after the blood is washed out by sterile saline solution run through the attached vessels is thoroughly macerated and ground up in a meat chopper. To every pound of placental tissue there is added one-half liter of sterile salt solution. This mixture is now allowed to infuse for forty-eight hours at refrigerator temperature, when it is filtered through a previously tested No. N Berkefeld filter for purposes of sterilization. To facilitate the filtration, the cylinder of the Berkefeld filter is filled with clean fine sterile sand until the candle is completely submerged. The clear amber-colored filtrate is then tubed or added to a two per cent sterile agar, which has been previously melted and cooled to about 41° C. The placental juice and the agar are then thoroughly mixed and the mixture sloped. The addition of three per cent glycerin possibly enhances its nutritive value."

ANAEROBIC BACILLI.

Ducrey, A.³² — Ducrey made attempts at cultivation from eight cases, which were as follows:

I. Tubercular-anesthetic type. — This case was the one from which Gianturco obtained his cultures. Ducrey obtained material from the bone marrow at autopsy.

2. Tubercular-anesthetic type. — Excised nodules.
3. Macular-anesthetic type. — Blood obtained by puncture of the skin.
4. Tubercular-anesthetic type. — Excised nodule from the face.
5. Macular-anesthetic type. — Blood obtained by skin puncture.
6. Tubercular-anesthetic type. — Excised cutaneous nodule during life and tissue from the spleen and bone marrow at autopsy.
7. Macular-anesthetic type. — This case had pemphigus, and material was obtained from the bullæ and blood by skin puncture.
8. Tubercular-anesthetic type — Three excised nodules on different occasions.

Technic: The skin nodules were excised under aseptic precautions. Blood was obtained after careful sterilization of the skin surface and allowed to drop into the culture medium. Bone marrow was obtained by cutting the long bones transversely, searing the ends and removing the marrow with a long platinum needle. Aseptic material was obtained from the spleen by searing the surface. The bacilli were abundant in all the tissues employed, but were not found in the blood in Cases Three and Seven, nor in the pemphigus bullæ of Case Seven. In Case Five a few bacilli were found in the blood. In Case Two no bacilli were found in the tissues, but masses of acid-fast granules, suggesting disintegrated bacilli, were found. The material from all these, with the exception of Case Four, remained sterile. This gave the culture described below.

The media employed were peptone broth, agar agar, glycerin agar containing five and ten per cent of glycerin, solidified beef blood serum, solidified hydrocele fluid and agar agar with grape sugar, as used by Campana. This medium has as its basis a broth made from equal parts of water and meat and containing 2.5 per cent peptone and .5 per cent of salt. To this broth one per cent agar and three per cent grape sugar are added. The tubes were inoculated by

deep puncture. They were then sealed in the blast lamp and incubated at 37° C. Growth appeared in seven days along the line of puncture in the form of a delicate line composed of minute colonies. Maximum growth was reached in fifteen to twenty days and the appearance then was that of a central line surrounded by a nebulous zone composed of exceedingly minute colonies, which had extended into the medium. The growth does not occur nearer the surface than two to three centimeters. Transplants gave growth in five to seven days. In ordinary agar agar, in glycerin agar, and in blood serum no growth takes place unless the medium is put in a vacuum. There was no growth on potato. In glycerin bouillon with three per cent grape sugar no growth was obtained until the tubes were placed in a vacuum, and then a good growth resulted. The broth remained clear, but there was an abundant granular deposit at the bottom and the sides of the test-tube, and a white surface pellicle. Gelatine at 20° to 25° C. gave no growth. Old grape sugar agar cultures kept for one year at 20° C. failed to give transplants in the usual stab cultures, but did when these cultures were placed in a vacuum. Ducrey concludes that his culture is a strict anaërobe. No motility was seen. Surface colonies were not obtained on any medium.

The morphology is given as a thin, straight or slightly curved rod, which may stain uniformly, but usually shows clear spaces and deeply stained granules. Small round forms occur in chains, and also coccoid forms in rosary arrangement and in groups packed like leprosy bacilli in tissues. No great morphological variation is described.

Staining reactions: The bacilli are Gram positive. They stain with aqueous methylene blue and methyl violet. They retain the stain by the Koch-Ehrlich method, but decolorize by the methods of Ziehl-Nielsen, Gabbett, and Baumgarten. Animal experiments with guinea-pigs and rabbits were negative; the animals were observed for nine months. No serum tests were made. Ducrey regards this culture as identical with that of Campana.

Campana.³³ — Campana's material consisted of excised leprosy nodules. These were introduced deep into five per cent agar media with peptone bouillon as a basis, and containing three per cent grape sugar. The reaction of the medium was neutral. The inoculated tubes gave anaërobic conditions, by virtue of the depth of the agar. They were incubated at 37° C. Cultures appeared in seven to nine days. No growth occurred in the presence of oxygen.

Morphology: The bacillus in length is equal to one-third to one-half the diameter of a red blood corpuscle (two to four μ). Its greatest width, when stained without drying, is one-fifth to one-eighth of its length. The rods are usually straight; sometimes curved. On staining, the protoplasm is homogeneous with or without clear spaces; no capsule could be demonstrated. The bacillus stains irregularly and shows bars and granules. Swollen segments may occur in the body of the bacillus or at one or both extremities. Sometimes the terminal swelling is club-shaped. In the cultures small round bodies are found, which resemble the granules found in the bacilli. These little rounded bodies are most abundant in old cultures and upon poor culture media. The bacillus is only slightly acid-fast, and decolorizes by the Koch-Ehrlich method and even dilute acid alcohol.

Campana³⁴ has readily obtained cultures in an agar medium containing meat peptone, two to three per cent; sodium chloride, one per cent; and grape sugar, one per cent. No growth was obtained in fluid media, even though all the oxygen was driven off by means of the passage of inert gases. The tubes were incubated at a temperature between 37° and 40° C. It is essential not to have great variations in temperature. The tubes were incubated under anaërobic conditions, but the method used is not given. Growth first appears as a linear clouding along the stab culture, deep in the agar, seven to nine days after inoculation. The line of growth is composed of minute colonies. The bacillus is an absolute anaërobe. Morphologically, the bacillus in the manner of its arrangement corresponds completely with the leprosy bacillus. The bacilli are straight or

curved rods, often somewhat shorter than the tubercle bacillus, and contain two to three or more deeply staining granules. These granules stain intensely and resist decolorization with ten per cent hydrochloric and nitric acids. Animal experiments were negative. Exact details are not given in this paper.

Serra.³⁵ — The material used consisted of excised tissue from living cases; seven in all. From three of these, all tubercular types, he obtained positive results. The method employed was that described by Campana and modified by the addition of fresh sterile guinea-pig tissue (liver, spleen, kidney, and heart) to the medium. Cultures were obtained in Campana's agar with and without the addition of sterile guinea-pig tissue and in peptone broth with the addition of sterile guinea-pig tissue. The broth contained two per cent glucose and two per cent of a ten per cent solution of sodium carbonate. The reaction was amphoteric. Serra states that when the broth is properly made it has a golden yellow color. The tubes were incubated under anaërobic conditions, and growth, both in agar and in bouillon, appeared in four to five days. On slanted solidified agar (agar, two per cent; glucose three per cent; peptone, one per cent; salt, .5 per cent, made from meat infusion and neutralized with ten per cent sodium carbonate) growth occurs about the tissue as a whitish film with a well defined corrugated border. In broth no clouding takes place, but a bluish white film forms around the side of the tubes, and a white deposit collects at the bottom; there is no surface growth. On shaking the tubes the growth is distributed throughout the broth in the form of fine granules, but it does not cloud. The anaërobic methods that were employed were the pyrogallic acid caustic potash method, the vacuum and deep stabs. Apparently the cultures grow well in simple deep agar stabs. Transplants to ordinary glucose agar produce visible growth along the line of puncture in seven to eight days, but only in the middle and lower thirds of the tube. By the fifteenth day the line of growth takes the form of a slender cord composed of

small colonies. The color is yellowish white. The colonies are compact, sharply defined, and flat. The only mention of color production in the colonies is that on glucose agar; on other media the color is white. Saccharose is not inverted, although the medium finally becomes acid. Indol is not produced. No product toxic for animals could be demonstrated in Berkefeld filtrates of broth cultures. The morphology is given as slender, straight or slightly curved bacilli, three to four μ long with rounded extremities. The arrangement of the bacilli in all media is in parallel groups. The bacilli do not stain uniformly, but show clear spaces and deeply stained granules. In old glucose broth cultures the bacilli are larger and clubbed forms are common; these were present, but less common in agar cultures. In old agar cultures, coccoid forms also occur. The bacilli are non-motile. Staining reactions: The bacilli are Gram positive. After staining with carbol fuchsin they resist decolorization with weak nitric acid solution (one to two per cent); with stronger solutions, only the granules remain stained and are brought out prominently as round and slightly ovoid bodies. The optimum temperature for growth is 37° C. Growth takes place slowly at 34° C. and as high as 40° C. The cultures resist a temperature of 45° C. for several days; 60° C. for three hours; direct sunlight five days; diffused light for fifteen days; putrefaction several days; one per cent phenol solution three hours. Inoculation experiments upon guinea-pigs, rabbits, dogs, and rats gave entirely negative results. Positive agglutination tests and complement fixation tests were obtained.

ACID-FAST NON-PIGMENTED BACILLI.

Weil, E.³⁶ — Weil claims to have obtained colonies which could not be transplanted from a case of tubercular leprosy. He lays emphasis on the choice of early lesions containing many living bacilli. His material was obtained by washing the surface of the lesion with ether, scraping away the superficial layers of the epidermis with a sharp sterile spoon, and plunging a sterile capillary pipette into the tissue, thus

securing a small cylinder of material rich in bacilli and free from blood. After trying out a variety of media he found the following the most favorable: A bouillon is made with five hundred grams of veal, seven hundred and fifty cubic centimeters of sea water, and two hundred and fifty cubic centimeters of distilled water. This is made decidedly alkaline, and there is then added forty cubic centimeters of glycerin, eight grams of glucose, ten grams of peptone, and two hundred and fifty grams of agar. To the melted agar, cooled to just below solidified point, is added one part of egg yolk to four of the medium. Colonies appear in five days at 37° C., and grow slowly for another fifteen to twenty days, when growth ceases. Transfers to different surfaces of the same tubes and to other tubes of the same medium do not give new colonies. By this method he obtained colonies eight times out of ten. Another method he employed which gave success two out of twenty-six times was in living fowl's eggs. The material was introduced aseptically by means of a capillary pipette into the yolk and incubated at 37° C. The appearance of the colonies is not peculiar. Growth took place equally well at a temperature between 37° and 39° C. Anaërobic conditions were not tried, although the growth inside the egg showed that free access of air was not necessary. The color of the colonies is pale yellowish; on egg agar the colonies are whitish; yellowish when seen against the white background of a less favorable agar serum medium, which Weil also employed. The colonies are firm in consistency.

Microscopically the bacilli have all the characteristics of the Hansen bacillus in tissues. They are acid-fast and resist decolorization with alcohol and ten per cent nitric acid. They are Gram positive and do not stain with basic methylene blue solution.

Karlinski.³⁷ — The material for cultures was excised tissue from nodular lesions. The culture medium was sterile blister fluid from leprosy patients obtained as follows: The skin was disinfected with soap, corrosive sublimate and alcohol.

The blisters were produced by means of freshly prepared Emplastrum Euphorbiæ. The blister fluid was withdrawn in sterile pipettes. After six to eight days' incubation at 38° C. a white sediment begins to be noticeable about the bit of tissue. In fourteen days compact masses, composed of acid-fast bacilli, are visible in the sediment and upon the upper surface of the fluid. Transferred to new blister fluid these masses enlarge slowly and crumble. Cultures were obtained in six successful generations during seven months. No growth was obtained on any other kind of serum, nor upon coagulated serum of leprosy patients. Growth does not occur below a temperature of 38° C. The bacilli are acid-fast in the first generation and partially so in the later generations. The morphology is that of the leprosy bacillus in tissues with occasional terminal clubbed swellings. The bacilli are non-motile. Animal experiments were entirely negative.

Marchoux, E.³⁸ — The material for culture consisted of nasal excreta, rich in acid-fast bacilli from leprosy patients. This material injected subcutaneously in white or gray rats produced an abscess in five or six days, and large masses of acid resistant bacilli were found extending out in long rope-like masses. This material was placed upon a large variety of media, under both anaërobic and aërobic conditions. Upon glycerin potato with ascitic fluid in one instance there developed a mixed culture of acid-fast bacilli. These bacilli grew out in long tresses or clusters. He also obtained impure cultures upon pieces of rat and pig spleen, which were sterilized at 110° C. and placed upon a medium of peptonized sugar agar. Marchoux does not insist that the acid-fast bacilli he cultivated were the leprosy bacilli. Acid resistant bacilli were also cultivated from cases of chronic rhinitis by the same method, but these decolorize much more easily than the bacilli obtained from leprosy. The latter resisted decolorization with three per cent hydrochloric acid in alcohol. He concludes that it is quite probable that the

impure culture he obtained was the bacillus of Hansen. Exact data are not given.

Twort, F. W.³⁹ — This is a preliminary note. The material used was the "nasal discharge and scrapings from a typical leper." The discharge contained large masses of leprosy bacilli and a number of contaminating microorganisms. Most of the latter were killed by placing the material in a two per cent solution of ericolin at 38° C. for one hour. Cultures were made from the sediment on different media and incubated at 38° C. All the ordinary laboratory media, including Dorset's egg medium, gave negative results. A successful medium was finally prepared as follows: tubercle bacilli grown on Dorset's egg medium were steamed and scraped off, ground up with glycerin and saline solution, and again steamed for half an hour. These tubercle bacilli were added in the proportion of one per cent to the mixed yolk and white of new laid eggs, seventy-five parts, eight per cent sodium chloride, twenty-five parts. The mixture was placed in test-tubes; "heated to 60° C. for one hour, and on the following morning incubated at 38° C. for six hours and again heated in water bath at 60° C. for one hour and set in slopes at 85° C." Upon this medium the acid-fast bacilli grew.

Microscopically, evidence of growth was obtained in about four weeks. Macroscopically, growth was apparent after about six weeks, and appeared as a colorless film along the needle track. Sub-cultures were always negative. No details have yet been published.

(The method of Twort is based on the fact that the glucoside ericolin kills many different kinds of bacteria, particularly bacilli of the colon group and micrococci. "The glucoside should be made up with distilled water in a two per cent solution. A lump of sputum is then put in a test-tube containing the ericolin and placed at 38° C. for three-fourths of an hour to one hour. Sub-cultures are then made on Dorset's egg medium and pure growths of tubercle bacilli will be obtained in from fourteen to twenty-eight days; the

tubes are sometimes contaminated with a few other organisms, chiefly tiny colonies of streptococcus and slow-growing colonies of organisms of the streptothrix group, but they are so few that they in no way interfere with the tubercle colonies, which can be easily sub-cultured.”)

Duval and Wellman.⁴⁰ — The authors call attention to a non-chromogenic acid-fast organism, which they find they have been cultivating along with the chromogenic variety. The following description is copied verbatim: “Source of culture: subcutaneous leprosy nodule. Date of isolation: Nov. 10, 1909. Morphology: Vegetative cells on amino-acid medium at 37° C. These vary from short plump to long slender rods. The chromatin is irregularly placed, often resulting in a beaded and bipolar effect. Clear non-staining areas are in consequence also irregularly disposed. Limits of size, two to five μ by .2 to .4 μ . Endospores absent; flagella absent; capsule absent. Involution forms: These may be seen in symbiotic cultures, in old pure cultures, and in cultures where the placental extract is diluted with other media. Staining reactions: Takes the ordinary basic stains, Gram positive and acid-fast.

“Cultural features: (1) On amino-acid agar stroke culture is of scanty growth, moist, filiform, flat, glistening, smooth, and somewhat opaque. Chromogenesis none. (2) In placental extract: surface growth none under ordinary circumstances (floats are at present writing being tried); clouding slight; sediment moderately abundant.

“Brief characterization: A Gram positive and acid-fast, non-motile, moderately slender bacillus growing only on a special media and generally presenting in culture the morphology of *B. diphtheriæ*.

“As to the inability of this bacillus to grow on ordinary media, it may be said that we have undertaken exact experiments to determine the minimum amount of amino-acid foodstuff (placental extract) necessary to grow upon solid media. Dilutions were made as follows:

"Placental extract, 1 part; 3 per cent sterile agar, 1 part.

" " 1 " nutrient agar, 2 parts.

" " 1 " " " 3 "

" " 1 " " " 4 "

" " 1 " " " 5 "

" " 1 " " " 6 "

" " 1 " " " 7 "

" " 1 " " " 8 "

" " 1 " " " 9 "

" " 1 " " " 10 "

"Transplants from the original medium (placental extract, one part; sterile agar, one part) to the other dilutions showed that the organism will grow upon one part in seven of placental extract with nutrient agar, but will not multiply upon one part in nine or ten."

Duval.⁴¹ — The author presents a new method of preparing media, which, however, is apparently not essential to success, as these media were not described in his earlier papers in which he records the isolation of the cultures now studied. The preparation of the media is as follows: "(a) two hundred cubic centimeters of fresh sterile human serum, egg albumin or the equivalent in grams of liver or placenta tissue is placed in a flask and twenty cubic centimeters of one per cent sterile physiological salt solution of trypsin is added. The mixture is now allowed to digest for five days at 37° C., changing the reaction to neutral with sodium carbonate solution from time to time, as the occasion demands. The contents of the flask are then heated at 70° C. for one hour and then filtered through a Berkefeld filter. A small quantity of the filtrate is tested for amino-acids (formol titration or bromic water test for tryptophane). (b) The same materials obtained under sterile conditions are autolized at 40° C. for two weeks, or hydrolized with a culture of Finkler and Prior's vibrio or some other bacterial proteolizer for twenty-four to thirty-six hours at incubator temperature and afterward neutralized with normal sodium hydroxide. The digested product is then filtered through a grade N Berkefeld filter, in order to clear and to sterilize. These filtrates

constitute the fluid culture media. To prepare the solid split-product medium, add an equal amount of any one of the above filtrates to agar 3, water 100, which has been previously tubed and sterilized. The agar is melted and cooled to 45° C. before adding the protein filtrate. It is now thoroughly mixed by proper manipulation of the tube, slanted and allowed to cool in this position. This constitutes the solid medium (amino-acid agar). Where it is desired to have other ingredients, such as glycerin, peptone, salt, they are added in the preparation of the agar. Plates may be prepared by pouring the amino-acid agar while liquid and allowing it to solidify."

Duval now has non-chromogenic acid-fast bacilli from eight cases of leprosy. These cultures are very specific in their growth requirements, and will not grow upon ordinary media. Inoculation experiments were done with the tissue from which cultures were obtained, in order to rule out the possibility of the presence of tubercle bacilli. All cultures came from excised cutaneous nodules from living cases: four yielded pure cultures of the acid-fast bacillus; the others gave impure cultures from which pure cultures were afterwards obtained. The initial multiplication of the bacilli in the tissue did not produce visible colonies, but on microscopic examination it was evident that growth had occurred. Sometimes colonies would spread out from the tissue in the initial culture.

Isolated colonies attained the size of small "pin heads;" they are elevated, colorless, and translucent. The growth is readily removed from the surface of the medium and when added to a liquid diffuses readily, presenting a uniform turbidity.

The morphology is given as of bacilli somewhat longer and thicker than the bacilli in human tissue. "The individual bacilli may vary in the same generation from solidly staining, short and long rods, with either pointed or swollen ends, to irregularly staining long and short, straight and slightly curved rods, whose chromatin may occur in isolated spherical masses, numbering from two to ten, and distributed

more or less evenly." The other forms noted are spindle-shaped bacilli with greatly swollen centers; very long forms which are often dumbbell and club shaped; branching, filamentous forms have not been seen. The bacillus is non-motile and does not produce spores. The staining reaction is given as completely acid-fast in comparison with the tubercle bacillus. Animal experiments: Two fully grown healthy rabbits were injected, one intraperitoneally, and the other intravenously, with large numbers of bacilli. One animal was killed six months and the other eight months after inoculation. No macroscopic lesions were found in either animal. The non-pathogenicity of this culture is recorded by Duval as evidence of its being the true leprosy bacillus, for the reason that leprous tissue does not produce lesions, while the chromogenic organisms recovered from the leprosy and other acid-fast bacilli do.

RAT LEPROSY CULTURES.

Dean⁴² failed to cultivate an acid-fast organism, although he used a large variety of media. From two infected rats he did obtain cultures at 37° C. of a diphtheroid bacillus, which, in the opinion of the author, resembles the cultures of Levy, Czaplewski, Barannikow, Teich, Babes, and Kedrowski. The colonies of this organism on solidified blood serum are indistinguishable from that of the Klebs-Loeffler bacillus. On nutrient agar and glycerin agar the growth is slower, the colonies are more delicate and more transparent than those of the diphtheria bacillus. On neutral and alkaline broth no pellicle is formed, but growth takes place along the test-tube wall and leaves a flocculent deposit at the bottom of the test-tube. In glucose broth the growth is granular, but not as abundant as that of the diphtheria bacillus. It forms acid in twenty-four hours, but not so strongly as the diphtheria bacillus. No visible growth appears on potato.

Morphology: In young cultures twenty-four hours old, on agar or blood serum, small diplobacillus forms are the rule, and sometimes the appearance is very like that of

Hoffmann's bacilli. "On the second or third day the bacilli become longer and take the appearance of diphtheria bacilli, in segmentation, club-shaped ends, etc., and later may form short, much-segmented branching filaments." In cultures two days old the club forms become very large, much segmented, like those of *Bacillus xerosis*.

Pathogenicity: The diphtheroid bacillus has a feeble pathogenic action on young rats. Three out of four young rats that received two cubic centimeters of a week's old broth culture died within a week. Old rats proved resistant.

Agglutination tests with this bacillus gave positive results with serum from a human case of leprosy, but not with normal human serum. The serum of rats infected with rat leprosy agglutinated the diphtheroid bacillus, but not the normal rat serum. Serum from a tuberculous human patient failed to agglutinate the diphtheroid bacillus.

Hollmann.⁴³ — The material used was blood from the heart of a rat with advanced lesions. Smears of the blood showed numerous acid-fast bacilli. The method was that of symbiosis, with amebæ and cholera vibrio, of Musgrave and Clegg. The inoculated tubes were incubated at 37° C. At the end of six days the bacilli were found to be longer, with well-marked, beaded, and occasional branching forms. The bacilli afterwards became much smaller than those observed in the tissues of rats, and in some cases were almost coccoid in form. When the number of bacilli showed an increase over the number originally found in the rat's blood they were transplanted to tubes of cholera vibrio with amebæ five days old. Smears made at periods from three to thirteen days showed evidence of multiplication of the bacilli. After the ninth generation the mixed cultures of amebæ, cholera vibrios and acid-fast bacilli were heated for thirty minutes at a temperature of 62° C. Transfers were made from the heated mixture to glycerin agar and incubated as before. In twenty-one days colonies became visible. They were raised, had smooth, irregular borders, and were pale yellow in color. Sub-cultures on glycerin agar grew well, "with a smooth,

raised, creamy-appearing growth of a lemon yellow color, which were found to be easily removed from the media with oese. On plain and nutrient agar the growth was of a lighter lemon yellow color, but not so profuse a growth as on glycerin agar."

The only comment on the morphology and staining reactions is that the preparations showed an acid-fast bacillus. Eight white rats inoculated with a suspension in saline solution after the third generation of pure culture developed lesions at the site of inoculation, in which acid-fast bacilli were found. In two of the rats there was consolidation of the lungs, and one of these consolidated lungs examined contained acid-fast bacilli. Three white rats were inoculated intraperitoneally. When chloroformed on the one hundred and sixteenth day no gross lesions were found, but smears made from the spleens of two of these rats contained acid-fast bacilli. In one rat smears from a nasal lesion contained acid-fast bacilli. Inoculations of two guinea-pigs, which were allowed to live for one hundred and ninety-seven days, were negative.

Chapin.⁴⁴ — The material employed was a "contaminated culture from the tissue of a leprous rat." The medium employed had the following composition: Egg yolk, one per cent; normal soda solution, .1 per cent; glycerin, .5 per cent; agar, two per cent; salt, .3 per cent. The egg yolk was added after the other ingredients were dissolved and sterilized. After the addition of the egg yolk, the medium was tubed and slanted, and after hardening was heated to 70° C. for one hour in the Arnold sterilizer. "The culture was kept most of the time in the incubator at 37° C. for a part of the time at room temperature, and transplanted about once a week. Upon making the original culture, and on each transplantation, the surface was moistened daily for two or three days with a one per cent solution of trypsin previously sterilized by passing through a Berkefeld filter." The acid-fast organism was isolated by plating upon the medium above described. The growth is

macroscopically dull, gray, brownish or yellowish, depending upon the medium and length of incubation. It overgrows the surface of condensation water, and does not emulsify readily. It grows fairly well upon similar media without trypsin. Upon bouillon it forms a pellicle.

"Microscopically, the organism is very polymorphous, depending upon the condition of cultivation, and forms are found resembling the leprosy bacillus in tissues as well as very short coccoid forms. Controls for sterility of the trypsin have usually remained sterile and acid-fast bacilli were never found. Similar attempts to grow the bacillus of rat leprosy without trypsin failed."

Zinsser and Carey.⁴⁵ — The material was obtained from infected rats. The method employed was that of tissue cultivation, developed by Harrison, Burrows, and others. The plants were made on cover slips over large, hollow, ground slides. The plasma was obtained in the usual manner by bleeding a young adult rat from the carotid artery into small paraffin tubes set in a brine ice mixture within large centrifuge tube holders. The tissue used was spleen from rats not over one week old. The procedure was as follows: "Minute drops of a thin emulsion of leprosy bacilli in salt solution were placed on the flamed cover slip. A very small piece of spleen tissue was placed on this and the whole covered with a large drop of plasma from a parafined pipette. These preparations were incubated at 37.5° C. and observed from day to day in a microscope chamber warmed by an electric bulb. In successful preparations migrations of large stellate cells usually began within twenty-four hours. The optimum growth was obtained at the end of forty-six days."

In two preparations out of many an unmistakable and very extensive increase of the rat leprosy bacilli had taken place by the end of two weeks' incubation. This increase in both cases occurred within large cells growing either separately or in small aggregations throughout the plasma. The bacilli were stained by carbol fuchsin and decolorized

with alcohol. In many instances cells containing great numbers of them retained their normal staining reaction. In others the containing cell was represented only by faintly stained granular detritus. No extra-cellular growth was obtained.

Bayon.⁴⁶ — The material was the spleen from a spontaneously diseased rat. "The media were those generally used for the cultivation of the tubercle bacillus." The colonies were ivory white, moist and of creamy consistency. Morphologically the bacilli are short; club shapes and branching forms are common. In general the microscopical appearances are very similar to that of the avian tubercle bacillus. Staining reactions: The bacillus is Gram positive. After staining with warm carbol fuchsin it resists twenty per cent sulphuric acid followed by alcohol.

The culture would not grow at room temperature nor under anaërobic conditions. No pigment was produced on any of the following media: fish juice glycerin agar, glycerin agar, glucose agar, glycerin potato, rice glycerin milk, sterilized wedges of rabbit's liver plus glycerin and Dorset's egg medium. All these media were suitable for growing the culture. Bayon says that "very numerous attempts" to cultivate the same bacillus from several other cases of rat leprosy failed completely.

Marchoux and Sorel.⁴⁷ — In excised lymph nodes incubated at 37° C. a marked multiplication of the bacilli was observed. Fragments of these lymph node cultures were placed upon pieces of rat spleen, previously sterilized at 110° C., and partially digested with trypsin. Minute colonies developed upon the surface of the spleen. There was no tendency to spread, and transfers to new rat spleen were fruitless. The original growth began quickly within eight days, and was very quickly destroyed. At the end of a month and a half the bacilli degenerated and became granular, at which time inoculation experiments were fruitless, which proved that the bacilli were dead. No mention is

made of the morphology and staining reaction of the bacilli in these cultures.

DISCUSSION.

It is unfortunate that the facts contained in many of the papers abstracted, and particularly the older ones, are not sufficient to make possible strictly accurate comparisons of the cultures. Practically all that is available for comparisons are: cultural appearances, morphology, staining reaction, and pathogenicity. The opinions of contemporaneous workers in regard to their cultures are possibly of some value, particularly when they conclude that their cultures are similar to those previously isolated. It is fairly certain that the cultures of Bordoni-Uffreduzzi, Babes, Gianturco, E. Levy, Spronck, Czaplewski, Teich, Kedrowski, Klitin, and E. Levy are identical, or very closely related.

A careful comparison shows that the cultures of these authors differ from one another only in minor respects: such as failing to give growth on bouillon and potato, or yielding only slight growth on these media. Klitin and Kedrowski claim that their cultures are pathogenic; the other authors obtained no results with animal inoculations. The available data for characterization of the diphtheroid organisms are as follows:

Colonies on media containing serum are yellowish white; on other media grayish white. The medium is not penetrated by the growth. The colonies on agar are round with serrated edges and elevated centers. The structure of the colony is granular and the periphery is translucent. The optimum temperature of growth is 37° C.; slight growth occurs at room temperature. Growth on potato and gelatine is very poor or negative. Bouillon remains clear when growth occurs, and usually takes the form of a surface pellicle with a granular sediment.

The morphology varies from a delicate, solidly staining bacillus to large irregularly stained diphtheria bacillus-like forms. The bacilli are partially acid-fast, after staining with carbol fuchsin. They take most of the ordinary aniline dyes,

and are Gram positive. Slight growth occurs in the absence of oxygen. Pathogenicity: none or questionable.

The culture of Bayon probably also belongs to the above group. It is doubtful whether the extremely pleomorphic organism described by Williams and the bacillus described by Duval as indistinguishable from *B. xerosis* belong to it. Of the acid-fast pigmented cultures it is questionable whether that of Rost is similar to those isolated by Clegg and by Duval. Of the anaërobic cultures it is necessary to conclude from the descriptions that those of Ducrey, Campana, and Serra are identical. In regard to the acid-fast non-pigmented cultures it is difficult to reach a decision. Duval and his associates are the only ones who have succeeded in maintaining transplants, with the exception of Karlinski, who grew them only in blister fluid. The very fact that Weil, Marchoux, and Twort did not get sub-cultures would indicate that they were dealing with the same organism that Duval has under cultivation, in the light of Duval's statements.

The fact that numerous workers situated in all parts of the world have encountered a variety of organisms with considerable consistency in leprosy must be regarded as significant. It is possible that we should not take into consideration the group of anaërobic bacilli, but there is no way of avoiding very serious attention to the significance of the presence of the diphtheroid group, the pigmented acid-fast group, and the non-pigmented acid-fast group. The number of times that each culture has been isolated is as follows:

Diphtheroids: Bordoni-Uffreduzzi, 1; Babes, 12; Spronck, 2; Gianturco, 1; E. Levy, 1; Czaplewski, 1; Teich, 4; F. Levy, 1; Barannikow, 2; Kedrowski, 3; Klitin, 4; Bayon, 1; Williams, 1 plus (?); Duval, 1. In addition a culture considered to be identical with Kedrowski's has been isolated in San Francisco by Professor Ophüls from an autopsy on a leper (personal communication). We have obtained a culture which answers in every respect to Kedrowski's bacillus from an excised epitrochlear lymph node from an advanced case of leprosy, which originally

came from the Cape Verde Islands. A complete account of this bacillus will be published later. The total number of recorded isolations in this group is therefore 37 plus.

Acid-fast pigmented cultures: Rost, 1 plus (?); Clegg, 15; Duval, 4. Total, 20 plus.

Anaërobic bacilli: Ducrey, 1 plus (?); Campana, 1 plus (?); Serra, 7. Total, 9 plus.

Non-pigmented, acid-fast cultures: Weil, 1; Karlinski, 1 plus (?); Marchoux, 1 plus (?); Twort, 1; Duval, 8. Total, 12 plus.

To draw conclusions from a review of this sort is very difficult. Indeed, any conclusions must necessarily be speculative, for a new technic and a few clear-cut facts can completely change the whole aspect of the subject. It is advisable, however, to discuss the facts accumulated with the hope of defining more clearly the problems still to be solved in the bacteriology of leprosy. First of all we must conclude that at least two, the diphtheroid and pigmented acid-fast, and possibly all four varieties of the bacilli are commonly found in leprosy tissue. The diphtheroid organisms have been found in all parts of the world; the pigmented acid-fast have been found most often in the Philippines and in Louisiana, independently and by competent bacteriologists. The non-pigmented acid-fast anaërobic bacilli perhaps have not been found often enough to have special importance. The prevailing opinions as to the nature of the leprosy bacillus, however, forces us to regard the few isolations of acid-fast, non-pigmented aërobic cultures as of extreme importance.

Granting the association of several different bacilli with leprosy there are two possible explanations. Perhaps the more plausible is that the organisms gain entrance through open lesions and are carried to various parts of the body, where they remain quiescent; it is difficult, however, to account for their presence in early lesions, whence several investigators have obtained each sort. More difficult to believe is it that these organisms are in actual symbiosis, and in this relationship are responsible for the disease, leprosy.

In favor of the first explanation is that diphtheroid bacilli and pigmented acid-fast bacilli are fairly generally distributed in nature. The former are frequently found on the surfaces of ulcerations, in skin lesions of all sorts, and upon normal mucous membranes. The latter, in the form of the smegma bacillus, are found on the surface of the body, and as the long list of acid-fast organisms now under cultivation testify, free in nature. However, diphtheroids cultivated from leprosy exhibit certain differences from those encountered from other sources, chief of which are partial resistance to acids after staining with carbol fuchsin, difficulty of staining with ordinary aniline dyes and more exacting growth requirements. A research into the nature of the bacteria infecting open lesions in leprosy is clearly indicated to help solve this point.

In connection with the pigmented acid-fast bacilli it must be insisted that the very carefully recorded experiments of Clegg and his associates and Duval and his associates admit of no other conclusions than that these organisms also come from leprosy tissue. Whatever their significance may be, the nature of the organisms, their free growth at ordinary temperatures and upon ordinary media, do not accord with our ideas of a parasite so highly specialized as the leprosy bacillus must be. It is difficult to understand why these cultures are so difficult to obtain in the first generation and so easy to maintain afterwards.

The possibility that the partially acid-fast diphtheroids may be converted into completely acid-fast bacilli must be taken into serious consideration, particularly in the light of Bayon's, Williams' and Kedrowski's recent publications, and because of the well known variation in staining reactions exhibited by leprosy bacilli in tissues (Babes, *loc. cit.*). The difficulty of obtaining leprosy bacilli in tissues stained with carbol fuchsin, followed by decolorizing agents and the ease with which they are stained with basic aniline dyes, for instance Unna's polychrome blue, are too well known to need comment. Unna⁴⁸ believes that the bacilli that take the counterstain are dead, chiefly because they are more numerous

in old lesions, but this is by no means proved. We do know that great variation in the resistance to acid decolorizing agents is shown by the tubercle bacillus after many years of cultivation. A fair review of this subject has been written by W. B. Wherry.⁴⁹ His own work was done with a tubercle bacillus culture, which was brought from Koch's laboratory in 1888. He has determined some of the conditions by which the cultures could be made either acid-fast or non-acid fast at will. Growth under conditions unfavorable to the synthesis of fats will make the cultures non-acid-fast. It is interesting to note that the morphological variation was extreme, from coccoid bodies to short or long, thick or thin, straight or curved rods, which were or were not acid-fast, according to the conditions of growth. Similar variations in morphology of tubercle bacilli from human and bovine sources, produced by change of medium, are recorded by Wolbach and Ernst.⁵⁰ These few facts indicate the possible claims for consideration of a theory, that, during the long residence in the body, the leprosy bacillus may develop strains exhibiting marked morphological and tinctorial differences, because of the variety of conditions to which they are subject, according to the character of the lesion and its location.

The value of animal experiments in the attempt to show specificity of organisms from leprosy is very questionable at the present time. The instances of successful inoculation of animals with leprosy bacilli direct from human tissues are few and not wholly beyond criticism. Sugai⁵¹ and Duval⁵² seem to have produced lesions in dancing mice by means of injections of leprosy tissues. Nicolle and Blaizot⁵³ give fairly conclusive evidence of having produced slight lesions in *Macacus rhesus* and *Macacus sinicus* by the injection of suspensions of bacilli from man. Stanziale⁵⁴ seems unquestionably to have produced active lesions in the anterior chamber of the eyes of rabbits, using tissue from very early leprosy nodules for inoculation. These few instances seem to be the only positive experiments recorded, although numerous workers have produced local abscesses in which

they thought multiplication of leprosy bacilli had taken place. On the other hand, inoculation experiments with cultivated organisms have been rather more successful. The pigmented acid-fast cultures isolated by Clegg and Duval have given positive results with guinea-pigs, dancing mice, rabbits, white rats, and monkeys. Regarding Kedrowski's experiments with the diphtheroid bacillus we can only call attention to the pertinent criticism of Babes. In general, all attempts to produce lesions with diphtheroid cultures from leprosy have failed. It is quite certain that animal inoculations up to the present time have yielded no evidence of value. Duval finds in his inability to produce lesions with non-pigmented acid-fast organisms an argument in favor of this culture being the true leprosy bacillus, in that the results are at least comparable with those obtained by injections of bacilli obtained directly from human tissues.

Further researches into the bacteriology of rat leprosy in the light of what has already been accomplished are to be awaited with great interest. Already we have the significant facts that Dean has cultivated a diphtheroid organism similar to those found in human leprosy; Hollmann an acid-fast bacillus producing lemon yellow colonies; Chapin an acid-fast bacillus producing dull gray colonies, which eventually become brownish or yellowish; and Zinsser and Carey an acid-fast bacillus in the cells of tissue cultures. Bayon's failure to repeat his cultivation of the creamy white acid-fast bacillus leaves cause for doubt as to the nature of this organism. The colonies that Marchoux and Sorel obtained on the rat tissue were not pigmented, but they were exceedingly minute and could not be transplanted. Should further researches bear out the apparent indications that similar varieties of organisms exist in connection with rat leprosy to those found in human leprosy much will be cleared up for both diseases, and the way towards the final solution of the bacteriology of leprosy may possibly be indicated.

The use of immune reactions, agglutinations and complement fixation for the purpose of establishing a relationship between bacteria isolated from leprosy and the disease, also

does not at present promise much enlightenment. The fact that positive agglutination tests have been obtained with human leprosy serum, and the following types of organisms, diphtheroid (Spronck and Babes), anaërobic non-acid-fast (Serra), pigmented acid-fast bacilli (Clegg and Duval), non-pigmented acid-fast bacilli (Duval) and with rat leprosy serum and diphtheroids (Dean) does not at the most necessarily mean anything more than that these organisms were present in the hosts from which sera were obtained. The complement fixation test has also not yielded reliable results by reason of the fact that so far no one has achieved a fixation test specific for any single acid-fast organism (Harris and Langford⁵⁵).

Complement fixation tests show an apparent relationship between rat leprosy and human leprosy in that in the great majority of cases the serum of lepers produces a marked fixation of complement in the presence of antigen made from tissue containing rat leprosy bacilli. Mezincesu⁵⁶ and Schmitt⁵⁷ obtained positive fixation tests with human leprosy serum and antigen prepared from tissue containing rat leprosy bacilli. The results were negative when controlled with human leprosy serum and the extract of normal rat tissues. The latter also found that a certain percentage of syphilitic sera gave positive results with rat leprosy antigen, just as the sera from cases of leprosy gives positive results with syphilitic antigen. Kritschewsky and Bierger⁵⁸ as a result of an elaborate series of complement fixation tests conclude that Kedrowski's bacillus is the true bacillus of leprosy, and that Duval's culture (chromogenic) is not specific for leprosy. The same tests show an apparent close relationship between the bacillus of tuberculosis and the leprosy bacillus taken directly from tissues and in culture (Kedrowski's culture).

It is quite possible, however, that eventually complement fixation tests may be made to distinguish the differences between closely related acid-fast bacteria, but at the present time conclusions cannot be drawn from the results of immune tests.

This review by no means gives a complete estimate of the work done in the attempts to cultivate the leprosy bacillus, for much negative work doubtless has not been published, and indeed a number of papers have been intentionally rejected by us. The whole, therefore, probably gives no adequate impression of the baffling nature of the bacteriology of leprosy.

The inevitable conclusion that a variety of bacilli in all parts of the world are fairly constant in the tissue of lepers makes the problem complicated, because it makes proof of the relationship of a single organism and the disease very difficult. The hope of producing progressive lesions in animals with bacilli obtained directly from human tissues should not be abandoned. The time of observation in most experiments recorded is probably too short. The similarities between rat and human leprosy, both pathological and bacteriological, are striking. In rat leprosy we have another instance of apparent absolute specificity of a bacterium for a given host in that the disease can be conveyed with certainty only to rats.

That human and rat leprosy may be due to more than one organism in symbiosis must be considered as a remote possibility in the solution of the problem. The possibility that the non-acid-fast and acid-fast organisms are related and may be converted one into the other is one that we must take, however reluctantly, into consideration, because of the great length of time that the leprosy bacillus resides in the human body and the variety of conditions it is subjected to by virtue of the stage of the lesion and anatomical situation. The loss and acquisition of properties as distinctive as that of acid-resistance are well known in the field of bacteriology, and indeed the changes undergone by the tubercle bacillus towards staining reactions is the strongest claim to consideration for this possibility.

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